Ultramicrostructural Study of Intravenous Fat Emulsion Using a New Fixation Method (I) A Comparative Study on the Effect of Fat Emulsion to Red Blood Cells Between Short Time Infusion of Intravenous Fat Emulsion and the One-Pack Infusion Method

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(I) A Comparative Study on the Effect of Fat Emulsion to Red Blood Cells Between Short Time Infusion of Intravenous Fat Emulsion and the One-Pack Infusion Method

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Introduction

Recently intravenous fat emulsion has been used clinically as an important source of calories[30] and essential fatty acids[1,32,37,38], and it is accepted as a safe infusate and commonly used. Although several adverse effects[4,14], including hematological abnormalities[5,19,36], such as anemia or enhanced coagulability[10,35] of platelets after using fat emulsion have been reported, the actual metabolism of fat emulsion has not yet been clarified.

On the other hand, the infusion technique termed total parenteral nutrition (TPN) or intravenous hyperalimentation (IVH) has been established to maintain nutritional status among those who are not able to take foods for a long term.

In conjunction with TPN or IVH, intravenous fat emulsion has become to occupy a great role in the nutritional management of these patients.

However, the adverse effects mentioned above have been still reported. Thus, the author attempted to investigate these adverse effects from the viewpoint of morphology. In this paper, in order to examine hematological abnormalities by fat emulsion, morphological observations of red blood cells were performed using transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

Materials and Methods

Subjects and Patients: Seven healthy adults (Blood types: type A(3), type B(1), type O(2), type AB(1)) were used as subjects for the in vitro studies. Thirteen patients were used for the in vivo studies on red blood cell.

Preparation of the Samples: Blood samples (6 ml) were taken from the subjects with a heparinized syringe. Each sample was divided into six 1 ml-samples, placed in a test tube, and washed

Key words: Intravenous fat emulsion, Malachite green, Echinocytosis, Intravenous hyperalimentation (IVH), Red blood cell membrane.
索引語：静注用脂肪乳剤，マラカイトグリーン，赤血球膜，中心静脈栄養，赤血球変性.
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three times with phosphate buffer (pH 7.25). Briefly, after the addition of the buffer, the sample was mixed gently with a pipette and centrifuged at 700 rpm for 5 min. After centrifuging, the supernatant was aspirated and a new buffer solution was added. This procedure was repeated two times except that the sample was not centrifuged after the last washing.

To 5 of 6 samples were added 0.1, 0.2, 0.5, 1, and 2 ml of fat emulsion (10% Intralipid®), respectively. The total volume of these blood, fat emulsion and buffer mixtures was brought up to 10 ml. As a control, Intralipid was not added to one of the samples. Each 2 ml of these mixtures and the control sample were placed in othe test tubes.

In Vitro Studies: Two trials (Groups 1 and 2) were performed; one with 2% glutaraldehyde as a pre-fixative, and the other with 2% glutaraldehyde and 0.1% malachite green according to our previously reported method[25,42].

Group 1: After preliminary preparation of the blood sample, 10 ml of 2% glutaraldehyde of the phosphate buffer (pH 7.25) was added to each of the 2-ml mixtures and mixed gently with a pipette. Thirty minutes after adding the pre-fixative, the mixture was centrifuged at 700 rpm for 5 min. The supernatant was aspirated and 1% osmium tetroxide was gently mixed in, and after an additional 1 hour, the mixture was centrifuged at 500 rpm for 5 min.

The resulting supernatant was aspirated, and 60% ethanol was added and centrifuged at 500 rpm for 5 min.

After repeating this procedure of aspiration and centrifugation, alcohol dehydration at increasing concentrations of ethanol to 100% was done. The resulting supernatant was aspirated and isoamyl acetate was gently mixed into the mixture. This procedure was also repeated two times at one hour intervals.

The subsequently fixed red blood cells were spread on a glass slide (5 mm × 5 mm) thickness 1 mm. These fixed cells then underwent critical point drying (Critical point dryer; HITACHI, Tokyo, Japan). Then, they were coated with Au ions (EIKO IB-3 model, Japan) and observed by SEM (HFS-2S and S-310, HITACHI, Tokyo, Japan). Some of the fixed cells were prepared for TEM with Epon 810 embedding and observed by TEM (HU-12A, HITACHI, Tokyo, Japan). For TEM observation, the finally centrifuged red blood cells were aspirated by a pipette and mixed with 10% melted gelatin. After having been hardened by glutaraldehyde, the samples underwent osmium tetroxide postfixation, alcohol dehydration, propylene oxide dehydration and Epon embedding.

Group 2: All blood samples were prepared in the same manner as in Group 1 except that, in addition to 2% glutaraldehyde, 0.1% malachite green was also used. As with Group 1, SEM and TEM observations were performed.

In Vivo Studies: Morphological changes in red blood cell were investigated after the infusion of fat emulsion in two trials (Groups 3 and 4) of patients.

Group 3: Six patients were infused with 500 ml of 10% fat emulsion (Intralipid) for 2 or 3 hours daily. The red blood cells were taken with a heparinized syringe before and after infusion and immediately washed with the phosphate buffer. As in the above groups, the samples were fixed for SEM and TEM observations.

Group 4: Seven patients underwent TPN according to the one-pack method of TANIMURA. Briefly, a daily menu of infusates were mixed in one soft bag which included fat emulsion, the fat emulsion is thus diluted and infused for 24 hours. Blood samples were taken by the same procedure as mentioned above, and fixed for SEM and TEM observations. Simultaneously other heparinized blood samples were taken from these subjects and submitted to Coil Planed Centrifuge (CPC)[8,9].

Differences between In Vitro and In Vivo Findings: *Groups 5, 6 and 7*: Blood samples were taken from healthy subjects and prepared by the same procedure as mentioned above. From each of the subjects, the mixtures were then divided into the following three groups.

Group 5: Solution of 2 ml of human albumin (25% albumin, Green Cross Pharm.) was added and mixed before adding the fat emulsion.

Group 6: Human serum from the same person was added and mixed before addition of the fat emulsion.

Group 7: Reversely, after adding and mixing 0.5 ml of the fat emulsion, albumin of each group was added.

The samples of these groups were prepared and observed by the same methods as mentioned above.

Results

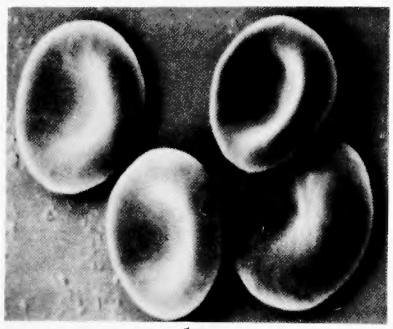
(1) *Group 1*: From SEM observations, it was noted that red blood cells developed, in vitro, echincytosis with the fat emulsion, and this phenomenon was dose-dependent between 0.1 and 2 ml (Fig. 1.). Though excessive volumes of the fat emulsion eventually cause hemolysis, fat particles of the emulsion could not be found in the sample which was mixed more than 3 ml of the fat emulsion. On the other hand, echinocytosis did not occur in the control group.

(2) *Group 2*: Slight echinocytosis was observed in the control group. This was thought to be due to malachite green. However, at this concentration (0.1%) of malachite green, no osmoral change in fixatives; this finding was confirmed by an osmometer. Also in the samples of this group, dose-dependent echinocytosis by fat emulsion was observed, and an attachment of fat particles to red blood cells could be observed by SEM (Fig. 2).

(3) *Group 3*: Echinocytosis was seen in red blood cells taken from patients receiving infusion of 500 ml of 10% fat emulsion for 2–3 hours, and it was most remarkable in the cells taken directly after the 2-hour finishing infusion. With time, this echincytosis diminished, but still continued for about 5–6 hours (Fig. 3). After several days of the observation, these red cells prior to infusion seemed to be normal morphologically. However, the evidence that the effect of the fat emulsion on the red cell membrane still exists even after more than 24 hours was confirmed by CPC (Fig. 4).

(4) *Group 4*: No changes were seen in the red blood cell in this group either before the starting of the one-pack TPN or during the treatment (Fig. 5).

(5) *Groups 5, 6 and 7*: The red blood cells showed echinocytosis in these groups, but it was to a lesser degree than that of Groups 1 and 2 (Fig. 6).



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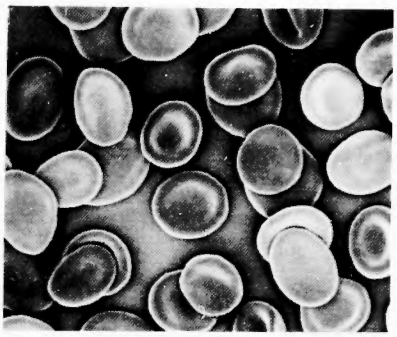

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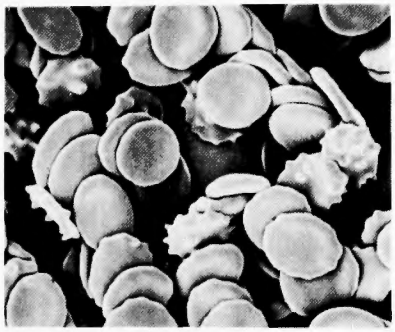

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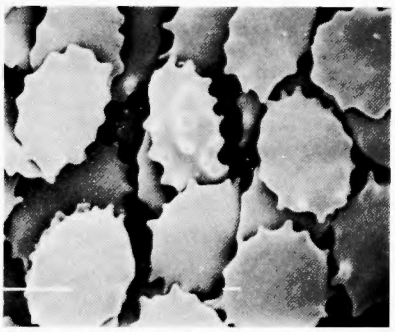

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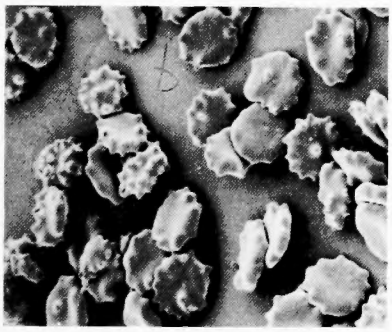

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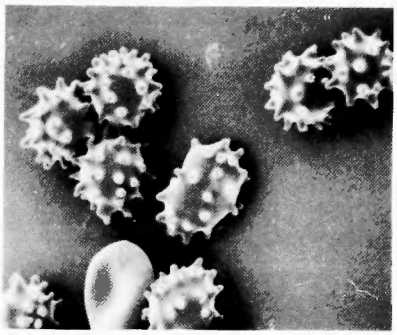

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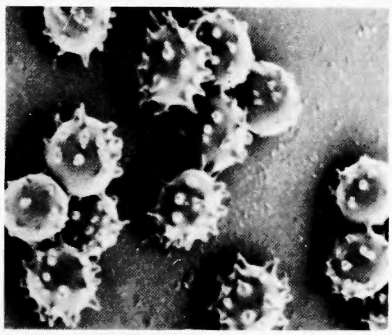
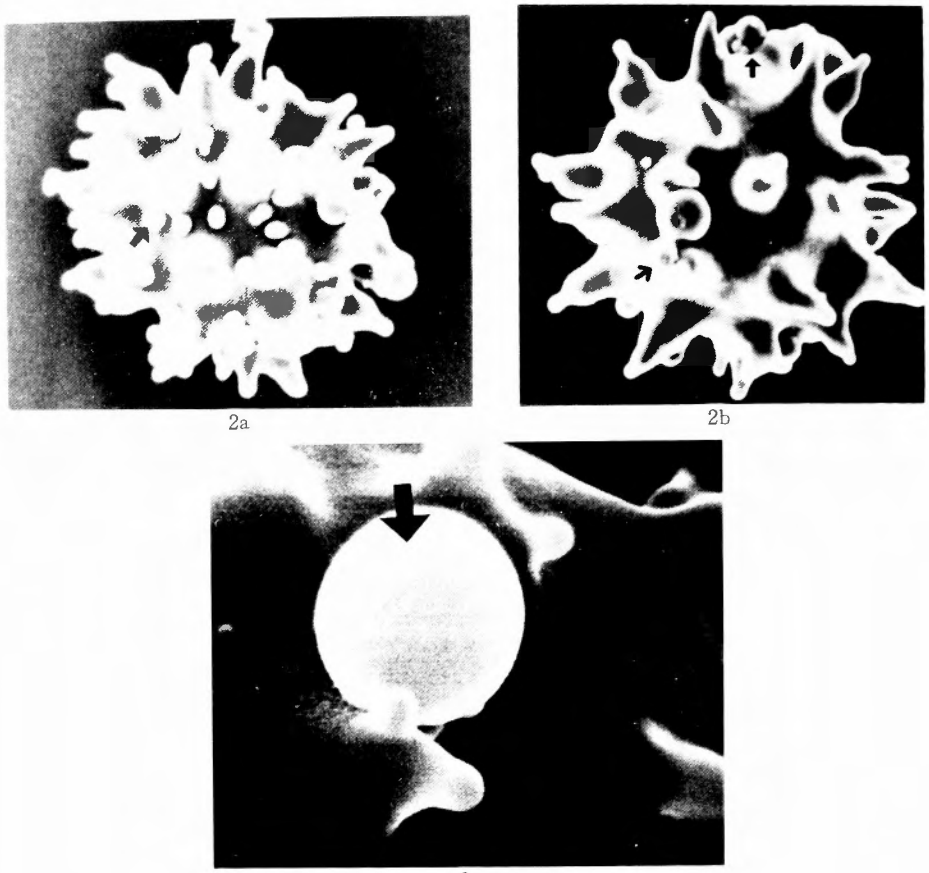


1g

Fig. 1. Human blood and fat emulsion.
Fig. 1a; normal blood cell without fat emulsion. Fig. 1b; 0.1 ml fat emulsion Fig. 1c; 0.1 ml, Fig. 1d; 0.2 ml, Fig. 1e; 0.5 ml. Fig. 1f; 1 ml and Fig. 1g; 2 ml.



2a 2b

2c

Fig. 2. Fat particles (arrows) attached to RBC. Prefixation was with glutaraldehyde and malachite green.

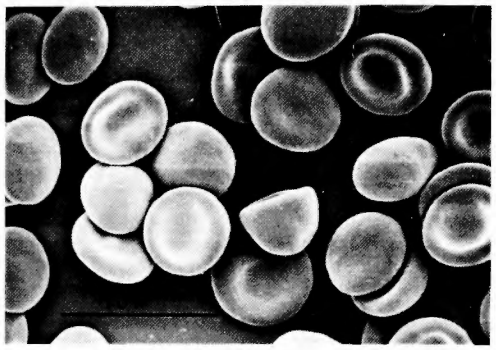
Discussion

A) Fixation Method of Fat Particles in Fat Emulsions:

By the usual fixation method, that is, prefixation-glutaraldehyde and postfixation-osmium tetroxide, only red blood cells were fixed and the fat particles were totally dissolved during alcohol dehydration. Therefore, it has been difficult to observe the relationship between fat emulsion and red blood cell. Moreover, in the fixing of the fat emulsion only osmium tetroxide has been used. However, even with this method, fixation is incomplete; all of these observations were done by only SEM.

In the usual fixation method, fat droplets in cells are not fixed or only incompletely electron pale upon TEM observation. Therefore, the fixation of extracellular fat droplets is highly unlikely. Needless to say, fat particles of the intravenous fat emulsion in a test tube of blood could not be fixed by this method.

KIMURA et al.[12,13] reported a method for observing intravenous fat emulsions. In their



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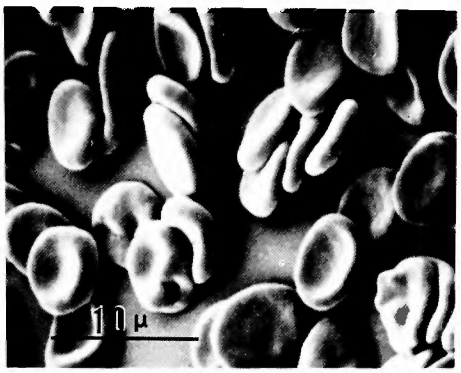

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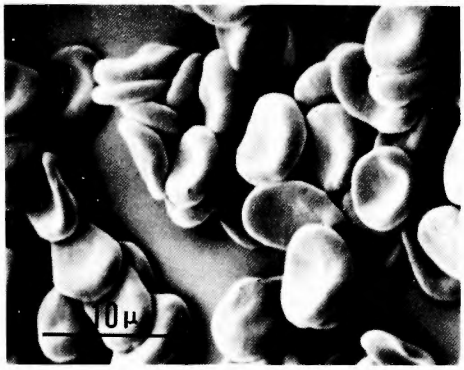

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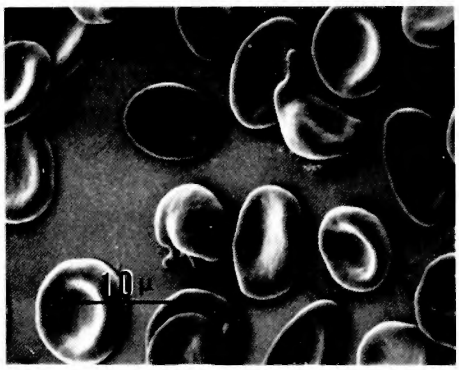

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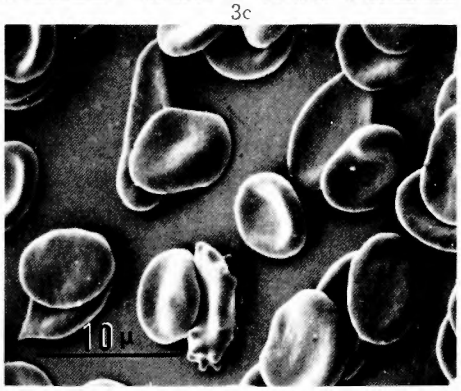

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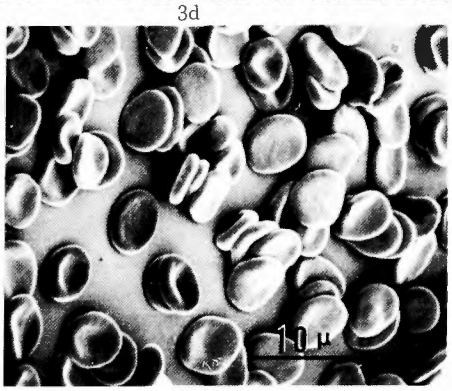

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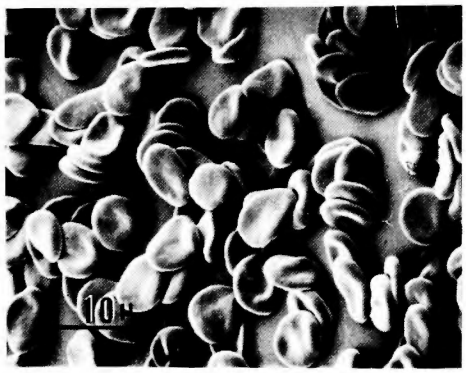


3g

Fig. 3. T. M. 28 y.o. male. Hematological change by infusion of intravenous fat emulsion by short time method.
Fig. 3a.; before infusion, Fig. 3b. and 3c.; 1 hour after cessation of infusion, Fig. 3d and 3e.; 2 hours after. Fig. 3f and 3g.; 5 hours after.

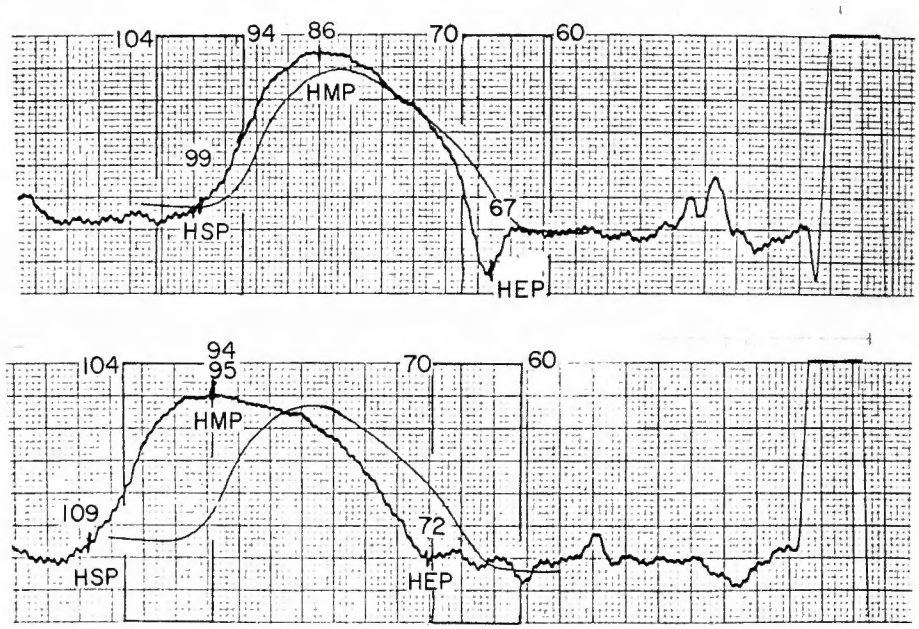


Fig. 4. CPC change before (upper) and after (lower) infusion of fat emulsion. Lower: 48 hours after cessation. By SEM observation RBC were normal.

method, fat emulsions were diluted with distilled water and a drop of the diluted emulsion was placed on copper grids covered with collodion membrane, and evaporated to dryness at room temperature. Then, they compared the size and shape of several types of fat emulsions using TEM.

A re-examination of this method was done by our laboratory, and the grids were observed using SEM. The results were as follows: there were no globular particles, and only unknown discoid material were observed. Though their method is among latest, it is not only inadequate,

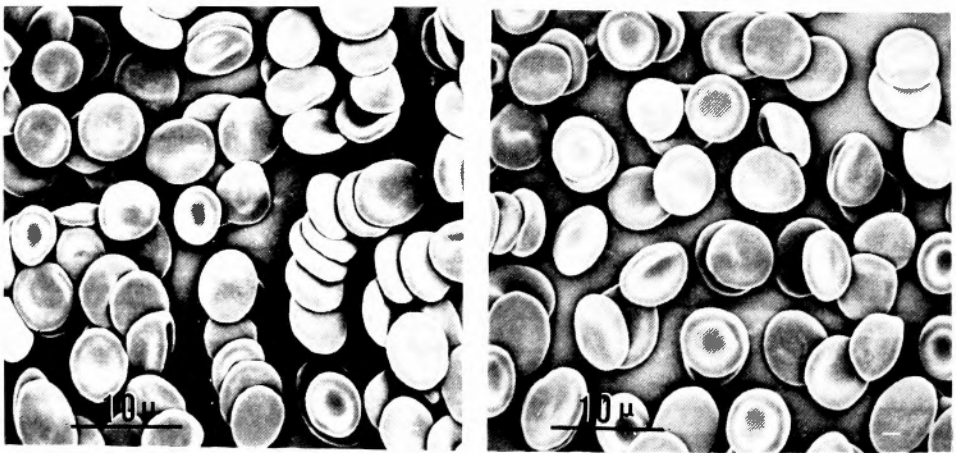
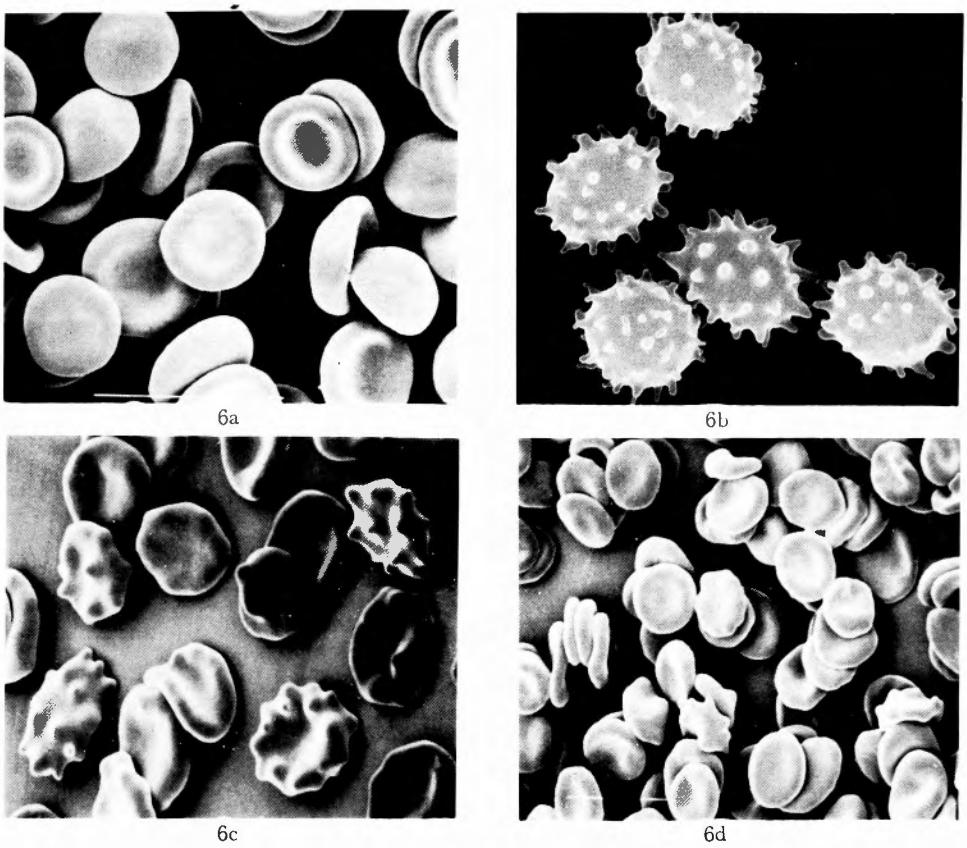


Fig. 5. Hematological change before (upper) and 48 after starting one-pack IVH (lower). G.Y. 61 y.o. male (carcinoma of the esophagus, preoperative)



6a 6b

6c 6d

Fig. 6. The role of serum against echinocytogenic effect of fat emulsion.
Fig. 6a.; before adding fat emulsion (normal RBC), Fig. 6b., RBC after adding fat emulsion (echinocytosis), Fig. 6c. and 6d. RBC after adding serum albumin (mild echinocytosis).

but also it lacks the theoretical background concerning the fixation of fat particles of the emulsion.

SCHOEFL[40] also recently reported a fixation method of fat emulsion (Intralipid) in which 2% osmium tetroxide is added for 40–70 hours at room temperature or at 4°C. The resulting sedimented pellet was broken up into smaller fragments and dehydrated with ethanol followed by Epon embedding. Though the subsequent TEM findings appeared to be far better than that of KIMURA et al, there may be considerable dissolution of fat materials by alcohol dehydration, and thus, this method also is inapplicable to either in vitro or in vivo studies. Therefore, as with the generally performed fixation method for TEM and SEM with a combination of glutaraldehyde and osmium tetroxide, this method adds little to the investigation of the metabolism of the infused fat emulsion.

TANIMURA[42] and the author[23,24,25] observed fat emulsions in a natural form by SEM using malachite green and glutaraldehyde (Fig. 7); the fat particles which had been already fixed did not dissolve during ethanol dehydration. On the observation of red blood cells by this method, a circular formation of particles (not particles of the fat emulsion) approximately 0.2 micra in diameter and with other smaller particles inside the formation was seen on the surface

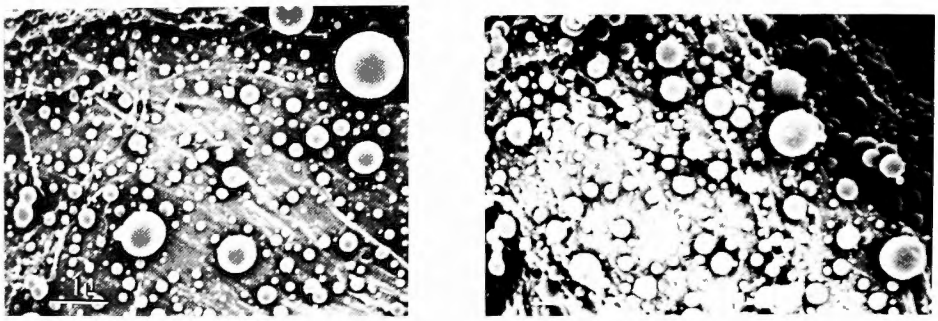


Fig. 7. SEM of fat particles on filter paper.

of red blood cell especially in the healthy subject group regardless of the blood type (Fig. 8). In the red blood cell which are mixed with fat emulsion, these circular formations disappeared with increasing volumes of fat particles. The fat particles and small particles in the circular formation presented very different shapes and structures by the examinations of SEM and TEM. By SEM, the fat particles appeared circular with smooth the surfaces, but the other small particles had highly irregular surfaces. By TEM, the fat particles were uniformly black, but the

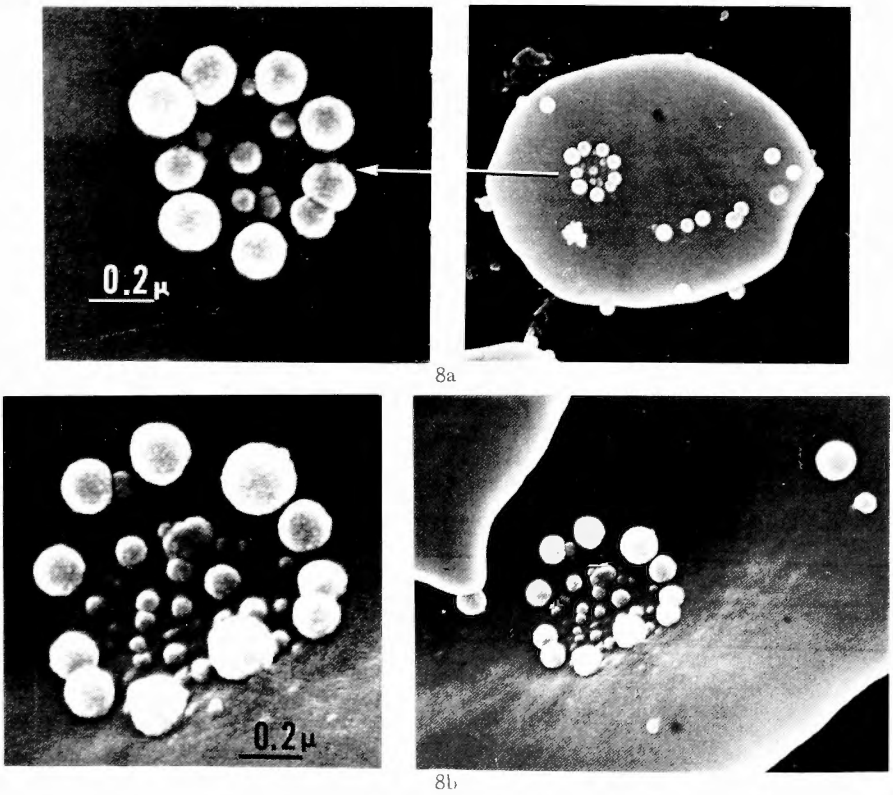


Fig. 8. SEM of circular formations on RBC of healthy human adults. Prefixation was glutaraldehyde and malachite green. Note the difference between these particles and fat particles. Fat particles were completely smooth.

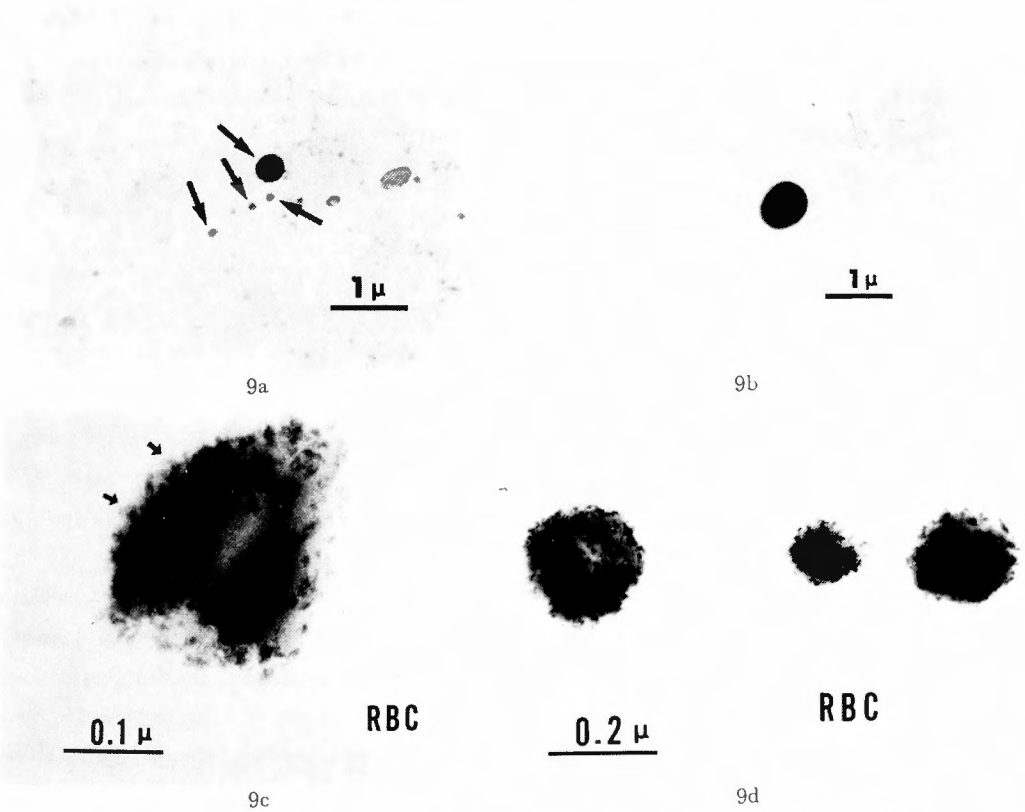
other small particles appeared have a core with branches (Fig. 9). The size of these particles were uniform and only one circular formation was thought to exist on each red blood cell. These particles were not found on the co-existing white blood cells or on the background slide.

As malachite green with glutaraldehyde and osmium tetroxide give strong density to fat materials, our findings indicate that these particles consisted mainly of fat materials, and are not artifacts during the procedure of fixation.

These particles with the circular formation began to disappear with increasing volumes of the fat emulsion suggesting that the fat emulsion can bring about significant changes in the structure of the red blood cell membrane.

B) *Effect of Fat Emulsion on Red Blood Cell Membrane*:

In order to detect substances on the red blood cell membrane several methods have been used; generally heavy metals are applied as a marker of compounds of the red cell membrane. On the other hand, malachite green is an organic compound without heavy metals. POULCHIO[31] concluded that malachite green gives fat materials strong electron density by uniting glutaraldehyde and osmium tetroxide. Though this is merely a hypothesis, the fact that fat particles were



9a 9b

9c 9d

Fig. 9. Comparision of fat particles and particles which formed circular formation of RBC.
Fig. 9a and 9b; TEM of fat particles, Fig. 9c and 9d., TEM of particles which formed circular formations.
All samples were without double stain.

given a strong electron density by our method strongly supports this hypothesis.

The existence of particles[22] as large as 0.2 micra in diameter has not yet been reported; usually materials which had been detected previously by heavy metal markers were far smaller. Thus some investigators are of the opinion that these particles are too large to be considered as markers of materials on the red blood cell membrane. However, on considering the special characteristics of malachite green the existence of these particles is highly possible.

Many studies have been performed on the effects of various agents[27] to red cell membrane, such as bile acids, substituted benzoates, diixopyrazplodines, vitamins, tranquilizers, anesthetics and antihistamines. Like the intravenous fat emulsion, these agents cause hemolysis at high concentration[9,17,21,28,39,41,13,44]. In many diseases the effects to red blood cell were reported on the bedside studies, such as liver cirrhosis, burn[6], hyperthyroidism[7], renal failure, and myotonic dystrophy[3], these disorders may not be thought directly related to the erythropoietic system. These investigations were made on the enzymatic[34] or ionic[33] level, however, their findings were not consistent.

C) Intravenous Infusion of Fat Emulsions

Since Fatgen,® sesame oil preparation, was first developed and used by HIKASA[13], several kinds of fat emulsions have been developed. Fatgen had been the first clinically usable fat emulsion. Recently, intravenous fat emulsion have been permitted by United States Federal Drug Administration. In Japan, several kinds of intravenous fat emulsions have been used. However, the contents of emulsion and production method are different each other.

Currently, intravenous fat emulsions are used clinically as an important source of calories and essential fatty acids. Fat emulsion is generally considered to be a safe infusate. Nevertheless, there have been several reports on the adverse effects of the fat emulsions and hematological adverse effects were noted among them. However, these findings have been concerned with the chemical or laboratory findings. Electron microscopical observation on the hematological changes has not yet been reported. This may be due to the difficulty in fixing fat materials for SEM and TEM.

Recently, we succeeded in easily and readily fixing fat particles of intravenous fat emulsion using malachite green for SEM and TEM, and this method was applied to the study of the ultrastructural morphology of the reaction between red blood cells and fat particles of the intravenous fat emulsion.

Malachite green is an organic compound which is thought that by combining specifically fat material electron dense material was formed in conjunction with glutaraldehyde and osmium tetroxide. Utilizing this advantage in fixing fat particles, observation of fat emulsion has been made easy.

The results of these studies showed that the intravenous fat emulsions cause echinocytosis[2,11,18,26,29] of red blood cells in vitro and in vivo. Therefore, these deformed red blood cells may be trapped by the reticuloendothelial system (RES)[15,16,20] increasing the possibility in the spleen of hemolysis. This effects was dose-dependent, thus the infusion of large doses of the fat emulsion eventually may cause hemolysis.

When the intravenous fat emulsion is infused via the peripheral vein, high concentrations of the emulsified fat and blood are mixed. The results of these studies indicates that a large number of red blood cells might be hemolyzed in a vein. Although most patients can receive intravenous fat emulsion clinically without remarkable side effects, an abnormal increase in serum hemoglobin level after the intravenous fat emulsion has been reported. Greater attention must be paid to infused patients who are critically ill of in the terminal stage of cancer.

Several adverse effects which were thought to be trivial in patients who are not critically ill were not always safe, because these patients were usually anemic and had hypoalbuminemia. If the fat emulsion is infused into these patients by the usual method, anemia will become more severe because the fat emulsion causes greater cell deformity, and the diminished low serum albumin levels will result in the fat emulsion attaching to red blood cells and thus increases the likelihood of red blood cell deformity. This study suggests that the fat emulsion should be infused by a one-pack method to minimize the effects against the red blood cell by diluting the fat emulsion over a 24 hours period.

When TPN is generally performed, fat emulsions are separately infused through the peripheral vein for 2–3 hours, but patients who often receive TPN are critically ill. Moreover, infused fat particles of the emulsion are quickly phagocytized by RES. In the case of short time infusion, macrophages phagocytized larger doses of fat particles. Blockade of bacterial phagocytosis after giving fat emulsions were reported.

With regard to echinocytosis of red blood cell in this study, other factors, such as pH, osmolarity and temperature were not echinocytogenic in the author's conditions, therefore echinochtogenesis was brought about by fat emulsion. There are many speculations concerning the mechanism of echinocytosis. Fat particles have a weak negative charge. But, by SEM observation, fat particles were directly attached to the red blood cells and these particles were enveloped by phospholipid. The attaching of phospholipid may cause changes in the structures of the red blood cell membrane.

Our findings of echinocytosis by fat emulsion and the disappearance of the characteristic circular formation of particles, though not performed on the molecular level, may add to our understanding of the mechanism of changes in the red blood cell membrane caused by various agents.

Conclusion

1. The intravenous fat emulsion was found to have a dose-dependent echinocytogenic effect on red blood cell in vivo; in vitro, excessive doses of fat emulsion caused hemolysis. The echinocytogenic effect was not seen when our one-pack method was clinically used. On the other hand, the commonly used infusion method of the fat emulsion caused echinocytosis. This difference may be of significance in the treatment of critically ill patients.

Serum albumin appeared to offer protection against the echinocytogenic effect of the intravenous fat emulsion, because the direct contact of fat particles to the red blood cell may be one of the causes of echinocytogenesis.

2. By using malachite green with glutaraldehyde for the pre-fixation of red blood cells, other particles in circular formation are formed on the cell membrane; they appear to consist mainly of fat materials and are almost uniform in size. The disappearance of these particles and the circular formation with increasing concentrations of the intravenous fat emulsion may cause significant changes in the red blood cell membrane; the investigation of these changes will help to clarify the mechanism of echinocytosis.

Acknowledgement

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和文抄録

新しい固定法による静注用脂肪乳剤の超微形態

（I） 脂肪乳剤の短時間投与法とワンパック方式投与法における赤血球に対する影響について

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静注用脂肪乳剤は効率のよいカロリー源として，又唯一の脂肪の非経口的な供給源として重要な輸液剤である．多くの実験によってその安全性が確かめられているが，形態学的には，今まで乳剤粒子の固定・観察が困難であったために十分な検討がなされていなかった．

著者は，脂肪と特異的に結合し，脂肪を有機溶媒に不溶性にし，かつ又高い電子密度を与えるマラカイトグリーンを用いて乳剤粒子を確実に固定し，その超微形態観察を可能にした．

この固定法を用い形態学的立場から脂肪乳剤の生体内での動態を観察し，脂肪乳剤の投与方法について検討した．

乳剤粒子は，投与後数時間血液中にあるため，赤血球に対する影響を経時的に観察した．

その結果脂肪乳剤は赤血球に対して in vitro で dose-dependent な echinocytosis を惹起せしめることがわかった．10％乳剤 500 ml を 2～3 時間で投与すると，in vivo でも軽い echinocytosis を惹起せしめる．しかし，脂肪乳剤を他の輸液剤と混合し，one-pack として濃度を下げて投与すると，かかる所見は観察されなかった．更に，血清アルブミンには脂肪乳剤の赤血球変形作用を防ぐ働きのあることが解った．

次に，脂肪乳剤を加えていない健常成人の赤血球をマラカイトグリーン＋グルタールアルデヒドで前固定をすると，赤血球膜上に 0.2μ 前後の均一な大きさをもつ粒子群が固定された．この粒子は SEM では直径約 0.8μ 前後の円として形成するのが観察されるが，それは TEM によると明らかに構造を有しており赤血球膜固有のものと考えられた．しかし，その機能などについては現在不明であるが，脂肪乳剤を加えていくことによって，この粒子が消失していくことから，乳剤粒子は赤血球膜に対して大きな影響を与えるものと考えられた．このような赤血球膜の変化は溶血や網内系による処理をうける原因にもなることが想像される．

以上のことから，特に，脂肪乳剤の投与が欠くべかざると思われるような重症の低栄養状態下にあるものでは同時に貧血，低酸素血症，低蛋白血症などがよくみられる所見であることから乳剤の投与に際しては慎重にそれを行なわなければならない．その点，濃度を下げしかも長時間かけて投与する one-pack 方式は従来の短時間投与法よりよりも好ましいと考えられる．